Back to the Reinnervation of the Pancreas After Transplantation? (Experimental Study on Dogs, Cats, and Rats)

D. Mikhalski[a], V. Coulic[b,*], D. Bilibin[c], V. Novikov[d], and P. Delrée[e]

[a]Digestive Surgery Unit, Hopital Erasme, Brussels, Belgium; [b]Laboratory of Experimental Medicine, Université Libre de Bruxelles, Centre Hospitalier Universitaire, Brugmann site Horta, Brussels, Belgium; [c]Neurophysiology Department, People Friendship University, Moscow, Russian Federation; and [d]Central Institute of Emergencies, Moscow, Russian Federation; and [e]Institut de Pathologie et de Génétique, Gosselies, Belgium

ABSTRACT

Background. Significant functional decrease and sclerosis of the pancreas graft in late delays cannot only be related to chronic rejection. Any transplantation leads to graft denervation, which may be an important cause of dysfunction. Studies concerning graft reinnervation were controversial.

Purpose of the Study. The purpose of this study was to investigate the feasibility and pertinence of a surgically directed reinnervation (SDR) of denervated/neuro-reflex isolated (NRI) or autotransplanted (aTx) pancreas.

Basic Procedures. Anatomy of the nerves penetrating into the pancreas was studied in humans, dogs, cats, and rats. Surgery and physiological investigations were performed in dogs, cats, and rats. Nervous conductivity between NRI, NRI+SDR pancreas, and brain was tested. Load tests with glucose, insulin, and adrenalin were performed; amylase and lipase were determined in fasted and not fasted animals to evaluate the influence of NRI and SDR on pancreatic function. Histology was provided. Observation delays were 6 months.

Main Findings. Anatomic feasibility of SDR in humans and animals was proved. Models of pancreatic tail NRI and surgical reconstitution of the interrupted nervous pathways (SDR) were elaborated in animals. The restoration of the pancreas-brain reflex axis after SDR was electro physiologically proved. As blood glucose curves after load test, exocrine amylase and lipase determination have shown that pancreas NRI or aTx leads to an exaggerated reaction to usual stimulations that may cause the observed graft functional exhaustion in late delays. SDR shortened the period of the graft neuro-reflex isolation, contributed to a quick normalization of its function, and prevented its late degradation.

Conclusion. SDR was shown to be a simple surgical technique, easily performed after the graft surgical revascularization. Its functional and morphological efficiency was tested and proved. Thus, SDR may be recommended in human pancreas transplantation as pertinent.

DURING the last years significant improvement of pancreatic transplantation results (organ as a whole) was achieved [1–4].

Nevertheless, some reports about functional status of the pancreatic grafts several years after transplantation have mentioned functional decrease and morphological sclerosis of the graft [5–7]. It was related to a chronic rejection process. But with the progress of immunotherapy [8,9] the rejection episodes are dramatically reduced and can no more be the only cause of the pancreatic graft dystrophy [10,11].

*Address correspondence to Very Coulic, Laboratory of Experimental Medicine, ULB, CHU Brugmann site Horta, 2, avenue Jean Crocq, 1020 Brussels, Belgium. E-mail: coulic.very@belgacom.net

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The pancreas organ transplantation procedure (as with any other organ grafting) includes only revascularization of the graft and reconstruction of the natural excretion ways [12–14]. So it leads to the nervous isolation of the grafted organ. At the same time the autonomic nervous system is known to regulate both endocrine and exocrine secretion of the pancreas, thus impacting glucose metabolism, as well as digestion processes [15–20].

In cases of extrinsic denervation of the pancreas, the basal pancreatic secretion in humans and the exocrine function in humans and dogs were significantly disturbed [15,21,22].

The question of the restoration of nervous connections between whole pancreas graft and recipient was discussed many years ago [23,24], concerned only spontaneous reinnervation, and never applied to clinics. The majority of authors agreed that the spontaneous growth of the recipient nerves into the graft occurs always very slowly.

Some investigations have shown that, in the case of the intestinal transplantation [25–34], spontaneous reinnervation is achieved too late to allow a complete recovery from the secretion, motility, and absorption dysfunctions caused by denervation. A surgical directed reinnervation of the intestinal transplant was proposed and tested; the conclusion was that it is feasible and efficient for fast penetration of the recipient nerves into the graft and it enhances the normalization of the graft function and morphology [26,35,36]. The idea of a surgical reinnervation of grafts was also proposed by other authors, particularly in the case of somatic nerve injury [37–40], but also for colon, kidney, and islet grafts with various results [41–44].

Hence, it seemed interesting to verify whether the denervation and reinnervation have the same influence on the function and morphology of the pancreas graft as was described in intestinal transplantation.

AIM OF THE STUDY

The aim of the study was to prove the feasibility and pertinence of the surgical directed reinnervation (SDR) of denervated or neuro-reflex isolated (NRI) or autotransplanted (aTx) pancreas for the optimization of the pancreas transplant condition.

The objectives were as follows: (1) on the basis of the study of the human and animal anatomy of the local pancreas innervation, to elaborate models of the NRI of the pancreas from the central nervous system (CNS) and spontaneous reinnervation, to elaborate a method of SDR of pancreatic grafts, (2) to evaluate the NRI influence on the endocrine and exocrine pancreatic function and morphology, (3) to evaluate the NRI + SDR influence on the pancreatic graft function and morphology, and (4) to justify the possibility of the surgical reinnervation of the human pancreas graft.

MATERIALS AND METHODS

The anatomy of the nerves penetrating into the pancreas was studied in 22 humans, 8 dogs, 9 cats, and 8 rats with the help of a binocular loupe MBC-3 with grid in millimeters. Taking into account the specific localization of both vascular sutures and possible nervous sutures during aTx in humans and in animals, the diameters of the dissected nervous fibers going to the pancreas were measured at the levels of the celiac trunk (level I) and at the origin of the splenic artery (level II).

Human cadavers were provided by the Department of Human Anatomy and Embryology of the Peoples' Friendship University of Russia (PFUR). Animal cadavers were provided by the PFUR animal house after euthanasia following local agreed protocol.

The surgical and physiological parts of the study were carried out in 27 dogs, 28 cats, and 94 rats, including fasted and nonfasted protocols.

All the animals used were hosted under standard conditions following "Guiding Principles for Research Involving Animals and Human Beings" Helsinki declaration of 1975.

All of the procedures were carried out under analgesia.

For surgery, general anesthesia was applied as follows in the dogs: induction by Aminosin (Chlorpromazine) 0.5 mL/kg and Droperidol 0.1 mg/kg (1–2 mL 0.005%) 15 minutes before the operation, followed by Thiopental Natrium 5% 0.5 mL/kg. After the operation, analgesia was pursued with intramuscular Analginum (Novalgin) 25% 1 mL.

For physiological study in the cats, intravenous Chloralose ((5ξ)-1,2-*O*-[2,2,2-Trichloroethylidene]-α-*xylo*-hexofuranose) 0.75 μg/kg (IV) was used.

In the rats, all invasive experiments were carried out under ether anesthesia.

Surgical Technique

NRI. Animals were placed in the supine position. After scrubbing and sterile draping, median laparotomy was performed.

The segment body-tail of the pancreas was dissected from all surrounding tissues in such a way that it remained attached to the splenic vessels and the pancreatic duct (Fig 1A). Dissection and hemostasis were performed using electro coagulation. The adventitia of the splenic vessels and pancreatic duct was accurately removed. In rats, residuary pancreas tissue attached to the duodenum was destroyed by electrocution, keeping the pancreatic duct in place. Abdominal cavity was closed without drain.

Pancreas aTx. This operation was performed just like NRI but with section and suture of the splenic vessels following the technique described by Roman RR et al [13,14]. The graft vessels were flushed with room temperature saline without heparin before orthotopic or heterotopic aTx. Warm ischemia time was 50 ± 5 minutes.

Exocrine pancreas secretion was drained into the jejunum by end-to-side suture between pancreatic duct and jejunum with running Catgut 4°°. In 4 dogs with segmental pancreas NRI and NRI + SDR, for the exocrine enzymes collection, pancreatic duct was sutured with the jejunum, a small patch of which was exteriorized to the skin as a modification of skin-intestine Thiry-Vella fistula.

SDR (During NRI or aTx Operations After Vascular Procedures). SDR consisted in paraneural and epineural suture with Prolen 10°° of the edges of the cut nervous branches surrounding the pancreas NRI/graft vessels with nerve trunks of the local neural plexus. For orthotopic reinnervation the nerve trunks surrounding either superior mesenteric artery and celiac trunk or splenic vessels were used. In heterotopic reinnervation the prepared nerve trunks of the graft splenic plexus were sutured with fibers of the hypogastric plexus (Fig 1B).

Control. The control groups in all animal models included animals after a laparotomy performed under general anesthesia followed by the pancreas surgical mobilization.

Follow-up. The animal care complied with the Guide for the Care and Use of Laboratory Animals [45].

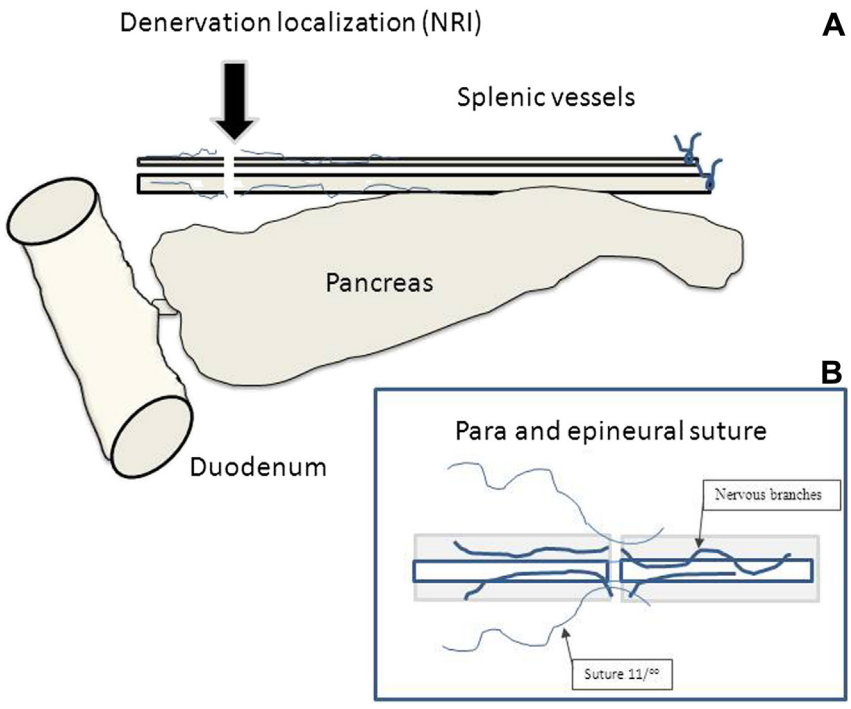

Fig 1. Schemas of operations NRI and NRI + SDR. **(A)** Isolation of the body-tail segment of the pancreas for NRI with reimplantation of pancreatic duct in the jejunum. **(B)** Technique of epi- and paraneural sutures for SDR

Taking into account the physiological character of the experiment, body weight and food intake were recorded daily for all of the animals. The rats were maintained in metabolic cages at a constant standard humidity and temperature, with a fixed 12-hour artificial light period.

Observation delays ran up to 6 months.

After animals were humanely killed, they underwent autopsy and light microscopy study.

Histological Investigation. Autopsy and biopsy samples taken within 1, 2, 4, 8, and 12 weeks after operation were fixed in formalin 10% solution then embedded in paraffin and stained using hematoxylin eosin and PAS (Periodic Acid Schiff) for light microscopy. This allowed appreciating the graft morphological condition at different stages in experimental series.

Registration of Electric Potentials in Response to the Stimulation of the Pancreas in Sensitive Zones of the Brain Cortex and Reticular Formation. To obtain a liable proof of the interruption of the neural connections between CNS and the pancreas the following technique was used. A first operation included the NRI or NRI with SDR (see above). A second operation was performed within 1 hour, 1 week, 1 month, 3 months, and 6 months after the first one and included a relaparotomy for the implantation of electric wires into intact and the NRI part of the pancreas, followed by a trepanation. With the help of stereotaxic navigation, electrodes were positioned in the cortex and reticular formation of the brain for detection and measurement of the reciprocal conduction between the CNS and pancreas head and body-tail.

The following parameters were chosen for the excitation of the neural tissue: stimulation current 12–18 μA, 0.5 μV, and 0.1 Hz. The registration was done by use of the "Multibasic OTC" Biomedical (Curno-Bergamo, Italy) programmed for 10-canal memory registration. The nervous connection was considered as established if the electrical stimulation of the pancreas induced a response signal enrolled in the cortex and reticular formation.

Load Tests with Glucose, Adrenalin, and Insulin. Blood glucose profile was chosen as the most representative target for evaluation of pancreatic endocrine function. Adrenalin and insulin were adopted as opposite regulatory factors of blood glucose level hormonal regulation. Disturbances of pancreatic function ought to modify the results of such load tests.

Load test was performed on rats to evaluate the influence of both NRI and SDR on the pancreatic function of blood glucose level regulation (Table 1).

Euglycemic clamps were performed on fasting and not fasted anesthetized rats.

Table 1. Functional Investigations and Number of Tests Per Series and Group

Series	Glucose Load	Insulin Injection	Adrenalin Injection
Control			
Fasted	5	5	5
Not fasted	6	6	6
NRI			
Fasted	11	11	11
Not fasted	11	11	11
NRI + SDR			
Fasted	11	11	11
Not fasted	11	11	11
aTX			
Fasted	5	5	5
Not fasted	5	5	5
aTX + SDR			
Fasted	5	5	5
Not fasted	5	5	5
Total animals	N = 75	N = 75	N = 75

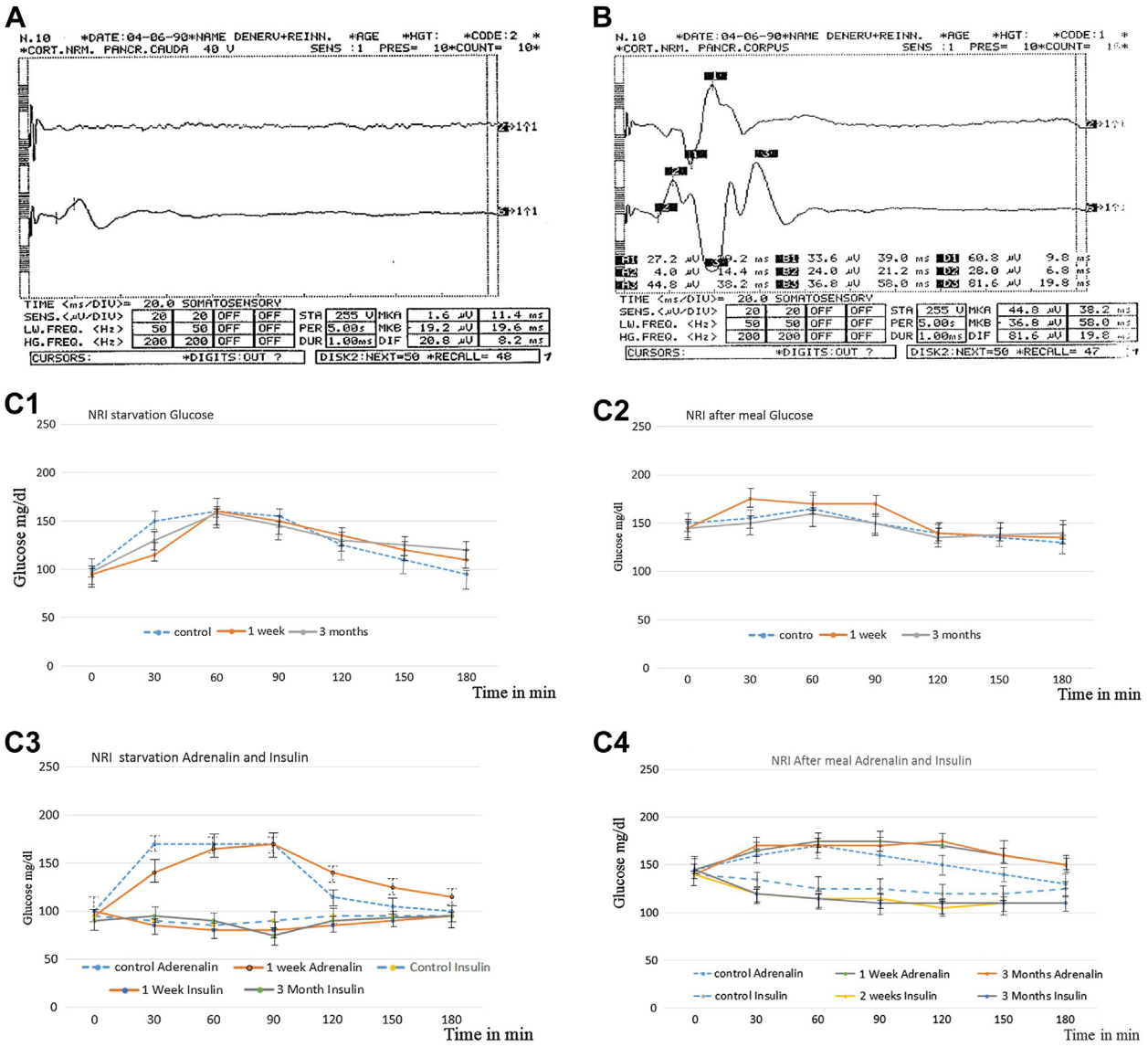

Fig 2. Functional investigations after NRI; NRI+SDR; aTx, aTx + SDR. **(A, B)** Results of electrophysiological investigations. **(A)** Day 8: no CNS response to stimulation after NRI + SDR of the pancreas tail. **(B)** Day 90: restoration of the response of CNS to the stimulation of the NRI + SDR pancreas tail. **(C, D)** Examples of load tests results. Abscise, time in 30-minute intervals after the load; ordinate, blood glucose levels in mg/dL. Plain lines: blue, curves at week 1; orange, at month 3; punt line, control. **(C)** NRI or aTx: 1 glucose load after starvation; 2 glucose load after meal; 3 adrenalin and insulin loads after starvation; and 4 adrenalin and insulin loads after meal. At every observation delay the blood glucose curve is significantly higher than the control one except for the first 30 minutes (delayed start of the reaction); at day 90 the curve is closer to control but still significantly different. **(D)** NRI + SDR or aTx + SDR: 1 glucose test after starvation; 2 adrenalin test after starvation; 3 adrenalin test after meal; 4 insulin test after starvation; and 5 insulin test after meal. In the case of SDR, as a rule, already after 1 month the difference between the control and SDR curves is no more significant in any point. **(E, F, G, H)** Results of exocrine amylase and lipase determination. Abscise, moments of investigation: 1 week, 1 month, and 3 months; ordinate, amylase level in IU. *P* was calculated relative to control. **(E)** NRI − starvation; **(F)** NRI after meal. At week 1 and month 1 after starvation, the amylase levels were significantly higher than in the control; at month 3, a significant decrease of amylase levels was observed, especially after starvation. **(G)** NRI + SDR − starvation, **(H)** NRI + SDR after meal. In NRI + SDR in both situations, starvation or after meal, at week 1, the amylase levels were significantly higher than in the control and the same as in NRI; at month 3 the amylase levels were not significantly different from the control and there was no sign of secretion decrease (difference between NRI and NRI + SDR amylase levels were quite significant).

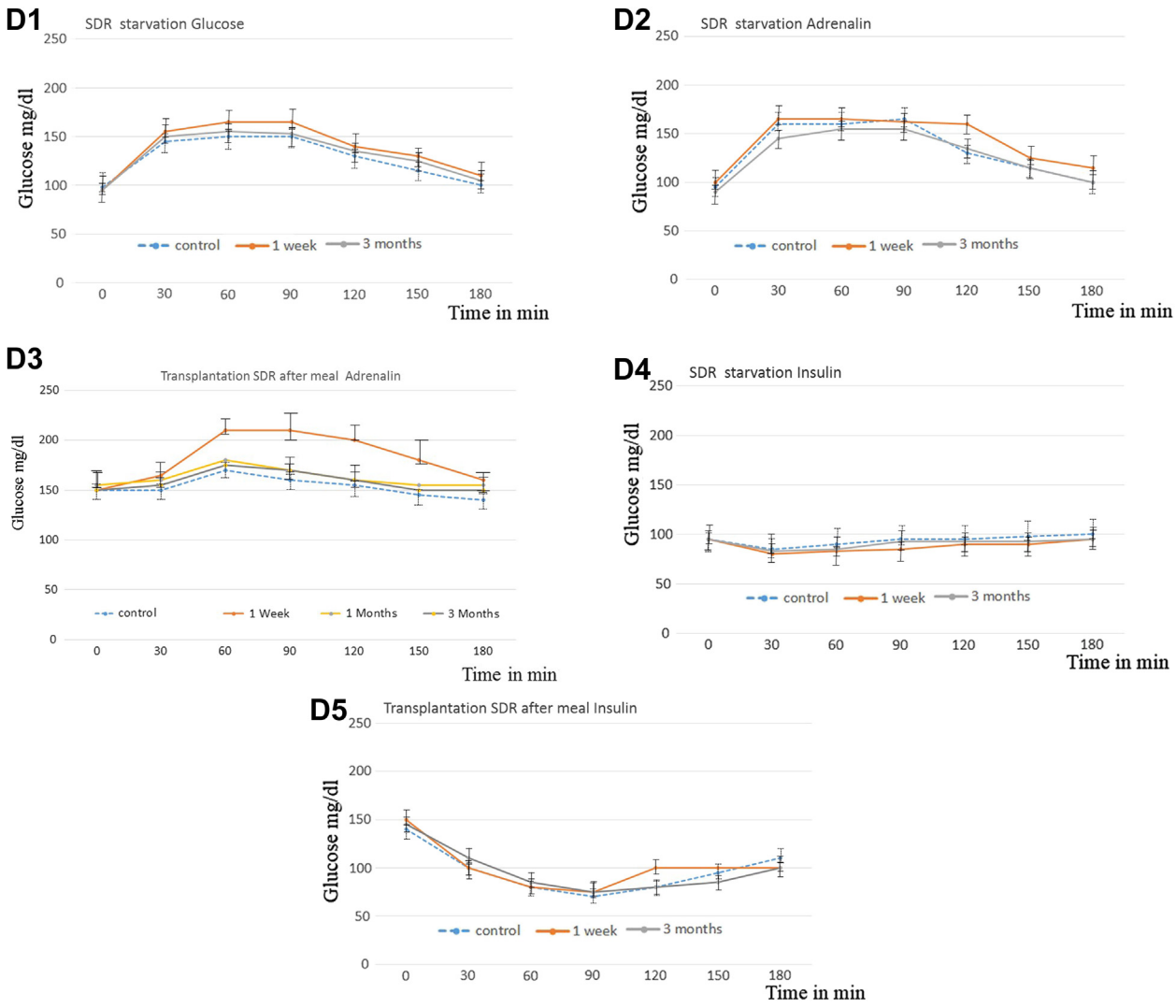

Fig 2. (*continued*).

The glucose solution (2 g/kg in 2 mL physiological saline) was given by injection in the stomach using a "heparin needle." Insulin ("Actrapid" Novo Nordisk, Paris, France) was injected subcutaneously 0.1 IU/kg.

Adrenaline (Epinephrine Sterop N.V., Brussels, Belgium) was injected IM (0.1 mL/0.1%/kg).

The blood samples were collected from the femoral vein after 24 hours fasting, water allowed, regimen (in metabolic cages) or in conditions of free intake of standard food.

Glucose level (mg/dL) was determined by the glucose-oxidase method using a glucometer (YSI Life Science, model 23 A, Yellow Springs, Ohio, United States).

The samples were collected at 0, 30, 60, 90, 120, and 180 minutes after the administration of glucose, adrenalin, and insulin.

Amylase and Lipase Investigations in Dogs and Rats. Amylase (IU/L) was measured by "Enzyline αAmylase PNP" set provided by bio-Mérieux company and lipase titrimetry (pH-stat).

(Amylase levels of pancreatic juice in healthy mature dogs are the same as in humans. Lipase enzymes levels in healthy mature dogs at the maximum limit of the normal range are around 325−810 IU/L. All in all, <500 IU/L is considered to be the normal range of lipase in canines. In rats, normality of blood amylase and lipase was given in control group).

In dogs, the investigations were performed at 1 week, 1 month, and 3 months after the operation. The amylase and lipase level determination was provided in 4 animals NRI and 4 animals NRI + SDR at the same time and the same conditions. The pancreatic excretion from skin-intestinel fistula (Thiry-Vella fistula) was collected in fasting (24 hours of food restriction) and immediately after a meal. The enzymes were determined in an aliquot of the juice volume, collected at intervals of 30 minutes during 3 hours.

In rats, the amylase and lipase level determination in blood serum was provided at 1 week, 1 month, and 3 months after the operation after 24 hours starvation and after glucose administration through intragastric administration of glucose at minutes 0 and 60. Analyses were performed in the Clinical Laboratory of CHU Saint-Luc Brussels.

Statistics Analysis. Results are given as mean ± standard deviation (M ± SD). Frequencies of categorical variables are given as percentages.

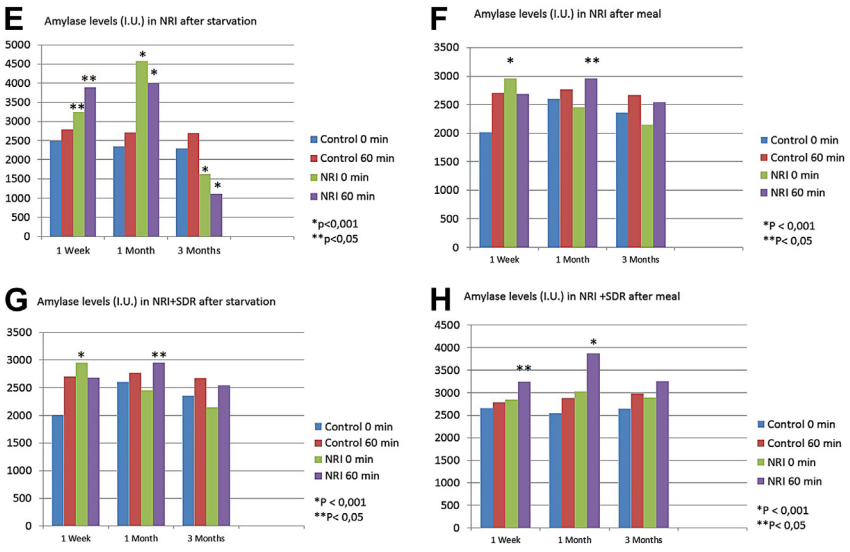

Fig 2. (*continued*).

Comparison of interval variables between the 2 groups was performed using Student independent samples *t* test.

For the evaluation of the functional test results the analysis of variance was used to compare multiple group means, followed by the Newman-Keuls test to determine statistical significance between 2 groups. When the data were not normally distributed, we used univariate and multivariate (Cox regression) analyses. Differences were considered significant at $P < .05$. All statistical analyses were performed on an IBM computer with PCSM software package (Personal Computer Software, Meylan, France).

RESULTS

The anatomical dissection of the different nervous trunks innervating the pancreas has shown that, in humans as well as in dogs, their diameters are $\geq$1 mm in 75% at both levels I and II. In the cats and rats they are $\leq$0.5 mm. As far as they run in a fascia sheet surrounding the blood vessels and split during the dissection of vessels before mobilization of the graft, for SDR it was proposed to suture the edges of nervous trunks via stitches between epineural layers or paraneural tissue close by the nerves themselves. In such a way an apposition of the cut edges was possible at level I and II in dogs, cats, and even rats (Fig 1B).

Electrophysiological investigation provided immediately after the suture has shown a temporary conduction of nervous impulse between stimulated denervated pancreas tail segment and brain RF was observed. At day 8 after NRI any conduction between NRI pancreas tail and the brain could

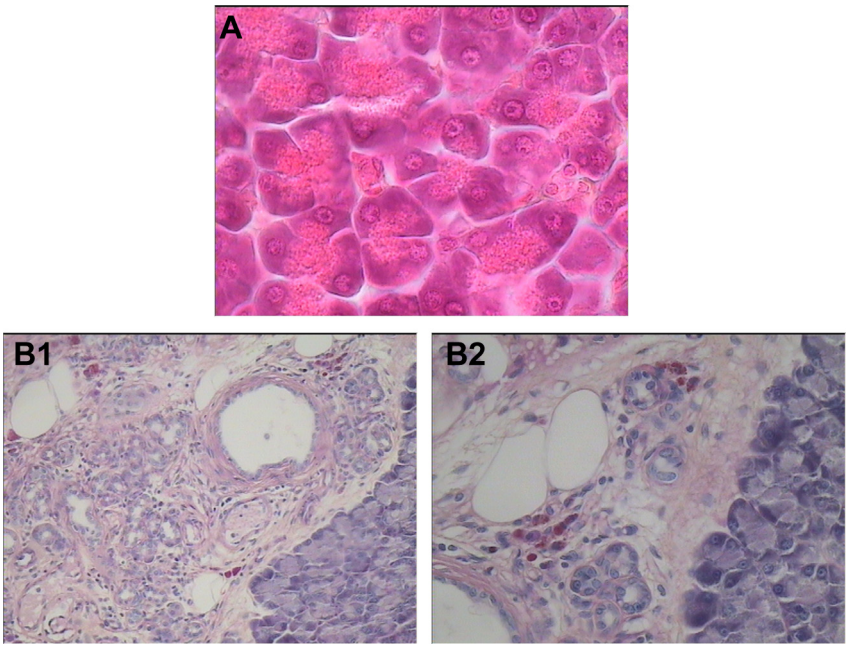

Fig 3. Histology of pancreatic graft 1 week and 3 months after aTx and aTx + SDR. At day 8 **(A)**, mild tissue congestion and increase of the nucleoli dimensions (hematoxylin eosin, original magnification $\times$40 and original magnification $\times$100); at day 90 **(B)**, normal structure and proliferation of the pancreatic ductuli (PAS, original magnification $\times$20 **(B1)** and original magnification $\times$40).

not be registered, whereas the impulses between intact pancreas head evoked a clear reaction in the RF and in the brain cortex. The situation remained practically the same up to 6 months after NRI, when a weak answer to NRI pancreas part stimulation was observed in the RF, but not in the cortex. After NRI + SDR the conduction was interrupted during the first weeks (Fig 2A) but within 1 month reaction of the RF to stimulation of NRI + SDR pancreas tail was noted. A complete restoration of the nervous conduction was observed at month 3 after NRI + SDR (Fig 2B).

Reactions of NRI or aTx pancreas to load tests in rats were characterized by a slightly delayed, slow beginning, an exaggerated development in intensity, and a prolonged duration. This reaction was the most evidently expressed in the series after adrenalin stimulation in fasted as well as in not fasted animals. An improvement was noted after 3 months, mainly in normalization of the response start, but there action remained significantly more intense ($P < .05$) and prolonged than in the control (Fig 2C).

In NRI + SDR, as well as in aTx + SDR, the same processes were observed ($P < .05$), but normalization began earlier (day 15) and was practically achieved at month 3: as a rule no more significant difference was noted between experience and control (Fig 2D).

The same rule was observed with exocrine secretion of amylase and lipase; their level in collected Thiry-Vella fistula pancreatic juice was significantly higher ($P < .05$) in NRI or aTx animals, than in control ones (Fig 2E and 2F). Even 3 months after the operation the situation remained the same, except that after starvation a significant decrease of secretion was to be noted.

After SDR in both experimental groups (NRI + SDR and aTx + SDR), the same phenomena were observed ($P < .05$) with a normalization of glycemic curves after the different load tests practically complete within 3 months (Fig 2G and 2H).

Histology has shown some discrete signs of edema in the early delays (8 days), proliferation of the small pancreatic ducts within the organ after 3 months, and signs of a mild fibrosis in late delays (6 months) after aTx. The structure of the exocrine and endocrine tissues of the pancreas was conserved (Fig 3).

DISCUSSION

The role of the interruption of nervous connections between organ grafts and CNS has been evoked since 1962 [25]. It has had sometimes unexpected consequences such as decrease of the body arterial pressure after "denervation" of kidneys or increasing of the liver regeneration potential after denervation due to vasodilatation of "desympathized" vessels [46–48].

The results of our investigations have shown that, as in intestinal transplantation, pancreas grafts seemed to follow the rule of Cannon and Rosenbluth [49,50], according to which denervated organs/structures mark an elevated sensibility to humoral stimulating factors. Delayed beginning, exaggerated intensity, and duration of answer to load tests testify in favor of this interpretation. Humoral transmission is slower than the neuro-reflex one, but compensatory increase of the sensitivity to humoral agents explains the increased intensity and duration of their influence. This was expressed either when using load tests as glucose, adrenalin, and insulin, or when investigating usual stimulation as food intake. A permanent increased functional solicitation may also explain the functional exhaustion of the organ in late observation delays.

Spontaneous reinnervation might be a waited, as far as the possibility of vegetative nerves and plexus regeneration was observed [51]. In our experiments, it seems to be slow and partial; there was no complete restoration of nervous conduction between the NRI part of the pancreas and the brain, partial improvement of the functional test results. It may be explained by the fact that, in case of NRI or aTx, the distance between the divided nervous trunks remains too important for the fast growth of the cut nerve terminals. Then spontaneous reinnervation occurs always late, maybe too late to be efficient. This corresponds to data in the literature concerning intestinal grafts [26], liver grafts [52–54], and even pancreas islet grafts [55–58], although here the situation is quite different, because vascular connections have not been surgically restored. Spontaneous reinnervation of other organ transplants and its enhancing effect on the graft functional welfare is poorly enlightened [59].

Taking into account that, in our test load experiments, even incomplete spontaneous reinnervation contributes to a slight but real improvement of the graft function, the proposed solution was to enhance this result by a surgically directed reinnervation, using suture or apposition of the divided nerves. Its application to intestinal grafts has been successful [26,35,36].

The doubtful conclusions given by some other studies, particularly concerning kidney graft SDR, may be due to the choice of functional criteria and a too short observation delay [43,44].

Our anatomic investigation has confirmed that SDR is technically possible. As performed in our experiments and reported in the few existing publications [26,35,36], SDR may lead to the restoration of the nervous conduction between grafts and CNS. The electric conduction through the nervous suture observed immediately after SDR can be explained by remained inviolate, vital activity of the distal axon before its following degeneration. Thereafter, the nervous conduction was indeed interrupted. But its restoration had already begun after 4 weeks and was practically complete within 3 months. This corresponds to the fact that results of load tests targeting both pancreas exocrine and endocrine function regulation were significantly improved already 3 months after operations and suggests that SDR has an important influence on the operated pancreas functional recovery.

Histological investigations have shown that NRI and aTX do not significantly affect the pancreas morphology, as also reported by other authors [7,14], at least in early delays. Perhaps ductuli proliferation after 3 months suggests a

compensatory reaction to functional tension? The tendency of fibrosis manifestation in the late observation period suggests the influence of cell overloading due to permanent excessive functional reaction to humoral factors.

Hence, our results suggest that SDR of the NRI or aTx pancreas also seems to be promising; enhancement of functional recovery and avoiding morphological "aging" of the graft were observed. The pertinence of SDR in pancreatic transplantation allows us to recommend this simple procedure for clinical testing. The coming back to the question of complete anatomic repair of all connections of grafts to recipients seems to be worthwhile now, at least for visceral organs, as far as the recipient's quality of life in the much delayed period after transplantation is considered.

In conclusion, anatomic studies have shown the feasibility of surgical reconstruction of the continuity of nervous plexus responsible for pancreas transplant/graft innervations. Models of pancreatic NRI and surgical reconstitution of the interrupted nervous pathways (SDR) were created and successfully tested in dogs, cats, and rats. NRI or transplantation of the pancreas has led to an exaggerated reaction to usual stimuli, which may be an important cause of the functional exhaustion of the graft in late delays. Spontaneous complete reinnervation was not evident. Electrophysiological studies performed in the cat models of NRI and NRI + SDR of the pancreas have proved the nerve conduction restoration after the proposed SDR.

Comparison of the results of load tests (glucose, adrenaline, and insulin) and amylase lipase determination in rat and dog models has proved the efficacy of SDR after pancreatic tail NRI or transplantation; SDR shortens the period of endocrine and exocrine dysfunction of the graft and prevents its late degradation.

The SDR is a simple surgical technique, easily and quickly performed after the graft surgical revascularization without any complication. Its functional and morphological effects were demonstrated as positive. Thus, SDR may be recommended to be used in human pancreas transplantation.

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